

**In the Specification:**

Please insert the paragraph beginning at page 1, lines 5, with the following paragraph:

-- This application is an application filed under 35 U.S.C. §371 which is based on International Application No. PCT/US00/15442, filed June 1, 2000, which claims the priorities of provisional applications U.S. Serial No. 60/137,213, filed June 1, 1999 and U.S. Serial No. 60/161,097, filed October 22, 1999, the contents of all of which are hereby incorporated by reference in their entirety into the present application. --

Please amend the paragraph at page 10, lines 14-21 to read as follows:

-- Fig. 17 shows the generation of  $\Delta$ Ad.AAV genomes ~~genenomes~~ by recombination between inverted homology regions. Recombination between two inverted repeats (IR) present in one Ad.AAV vector. The first-generation Ad.AAV vector (~34kb) contains two 1.2kb inverted homology elements flanking the transgene cassette. One AAV-ITR is inserted between the Ad packaging signal ( $\psi$ ) and the left IR. During Ad replication, recombination between the Irs mediates the formation of the  $\Delta$ Ad.AAV genomes with the transgene flanked by Irs, AAVITRs, Ad packaging signals, and Ad ITRs. These genomes are efficiently packaged into Ad capsids. Figure 17A shows recombination between two Ad.AAV vectors each having an intact transgene sequence. Figure 17B shows recombination between two Ad.AAV vectors having either a partial 5' or partial 3' portion of a transgene sequence. --

Please amend the paragraph at page 10, lines 23-31 and continuing at page 11, lines 1-3 to read as follows:

-- Fig. 18 shows the structure of ~~Ad5/41~~, Ad5/35. Schematic diagram of the original E1/E3 deleted Ad50based vector with GFP-expression cassette inserted into the E3

region (Ad5GFP) and the chimeric vector Ad5GFP/F35 containing the Ad5/35 fiber gene. The 2.2kb Ad5 fiber gene was replaced by a 0.9kb chimeric fiber gene encoding for the short shaft and knob of Ad35 by a technique that involved PCR-cloning and recombination in E.col. Kpn I (K) and Hind III (H) sites localized within or around the fiber genes are indicated. The lower panel shows the detailed structure of the chimeric fiber region. The Ad5 fiber tail [amino acids (aa): 1-44] were joined in frame to the Ad35 fiber shaft starting from its first two amino acids (GV), which are conserved among many serotypes. A conserved stretch of amino acids TLWT marks the boundary between the last  $\beta$ -sheet of Ad35 shaft and the globular knob. The Ad35 fiber chain termination codon is followed by the Ad5 fiber poly-adenylation signal. --

Please amend the paragraph at page 11, lines 5-28 to read as follows:

-- Fig. 19 shows the cross-competition for attachment and internalization of labeled Ad5GFP, Ad35, and chimeric Ad5GFP/F35 virions with unlabeled viruses, and with anti-CAR or anti- $\alpha_v$ -integrins Mab. (A) For attachment studies,  $10^5$  K562 cells were pre-incubated with a 100-fold excess of unlabeled competitor virus at 4°C for 1 h. Then, equal amounts of [ $^3$ H]-labeled viruses, at a dose equivalent to an MOI of 100 pfu per cell determined for Ad5GFP, were added to cells followed by incubation at 4°C for 1 h. Cells were then washed with ice-cold PBS, pelleted and the percentage of attached virus (cell-associated counts per minute) was determined. For analysis of cross-competition for internalization, cells were pre-incubated with a 100-fold excess of competitor virus at 37°C for 30 min before labeled virus was added. After an additional incubation at 37°C for 30 min, cells were treated with trypsin-EDTA for 5 min at 37°C, washed with ice-cold PBS, pelleted, and the percentage of internalized virus was determined. For controls, cells were incubated with labeled viruses without any competitors. Preliminary experiments had shown that the conditions chosen for competition studies allowed for saturation in attachment/internalization on K562 cells for all unlabeled competitors. (B)  $10^5$  K562 cells were pre-incubated for 1 hour at 4°C with anti-CAR MAb (RmcB, diluted 1:100) or with

anti- $\alpha_v$ -integrin MAb (L230, diluted 1:30), followed by incubation with labeled viruses according to the protocols for attachment or for internalization as described above. For each particular serotype, the percentage of attached/internalized virus was compared to the control settings, where cells were preincubated under the same conditions with a 1:100 dilution of an irrelevant antibody (anti-BrdU Mab) before addition of the labeled virus. Note that the specific competitors but not the corresponding controls significantly inhibited Ad5 internalization to a degree that is in agreement with published data (59).  $N \geq 4$ . (C) In internalization studies, Ad5 did not inhibit internalization of Ad35 or Ad5GFP/F35 into K562 cells. (D) In internalization studies, L230 monoclonal antibody did not inhibit internalization of Ad35 or Ad5GFP/F35 into K562 cells. --

Please amend the paragraph at page 11, lines 30-31 and continuing at page 12, lines 1-6 to read as follows:

-- Fig. 20. Cross-competition for attachment and internalization of [ $^3$ H]-labeled Ad5GFP, Ad35, and chimeric Ad5GFP/F35 virions with unlabeled Ad3 virus (A), and of [ $^3$ H]-labeled Ad3 virions with unlabeled viruses (B).  $10^5$  K562 cells were pre-incubated with a 100-fold excess of unlabeled viral particles according to attachment or internalization protocols described for Fig.6. Equal amounts of [ $^3$ H]-labeled Ad5GFP, Ad5GFP/F35, or Ad35 (A) or [ $^3$ H]-labeled Ad3 (B) were added to cells at a dose equivalent to an MOI of 100 pfu per cell for Ad5GFP. In control settings, cells were incubated with labeled viruses without any competitors.  $N=4$ . (C) In attachment studies, Ad35 does not significantly inhibit attachment of Ad3 to K562 cells. (D) In internalization studies, cells pre-incubated with Ad35 significantly inhibit internalization of Ad3. --

Please amend the paragraph at page 12, lines 14-24 to read as follows:

-- Fig. 22 shows the distribution of GFP-positive cells in subpopulations of human CD34<sup>+</sup> cells expressing CAR or  $\alpha_v$ -integrins.  $1 \times 10^5$  CD34<sup>+</sup> cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu/cell ~~as described for Fig. 8~~. Twenty-four hours after infection, cells were incubated with anti-CAR (1:100 final dilution) or anti- $\alpha_v$ -integrin (1:30 final dilution) primary MAbs for 1 h at 37°C. Binding of primary antibodies was developed with anti-mouse IgG-PE labeled secondary MAbs (1:100 final dilution) at 4°C for 30 min. For each variant,  $10^4$  cells were analyzed by flow-cytometry. The mock infection variants represent cells incubated with virus dilution buffer only. The quadrant borders were set based on the background signals obtained with both the GFP- and PE-matched negative controls. The percentages of stained cells found in each quadrant are indicated. The data shown were representative for three independent experiments. --

Please amend the paragraph at page 12, lines 26-31 and continuing at page 13, lines 1-18 to read as follows:

-- Fig. 23A-23B shows the distribution of GFP-positive cells in a subpopulation of human CD34<sup>+</sup> cells, expressing CD34 and CD117 (c-kit). (A) Co-localization of GFP expression with CD34 or CD117: CD34<sup>+</sup> cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu per cell under the conditions ~~described for Fig. 8~~. Twenty-four hours after infection, cells were incubated with anti-CD34 PE-conjugated MAbs (final dilution 1:2) or with anti-CD117 PE-conjugated MAbs (final dilution 1:5) for 30 min on ice, and  $10^4$  cells per variant were subjected to two-color flow cytometry analysis. For negative control staining, no antibodies were added to the cells before analysis. The mock infection variants represent cells incubated with virus dilution buffer only. The quadrant borders were set based on the background signals obtained with both the GFP- and PE-matched negative controls. The percentages of stained cells found in

each quadrant are indicated. The experiment was performed two times in triplicates, and typically obtained results are shown. The SEM was less than 10% of the statistical average. (B) Transduction of CD34+/CD117+ cells with Ad5GFP and chimeric Ad5GFP/F35 virus vectors: CD34+ cells, cultured overnight before staining in media without SCF, were incubated with PE-labeled anti-CD117 MAb for 30 min on ice. The fraction of CD117-positive cells was sorted by FACS. More than 97% of sorted cells were positive for CD117.  $1 \times 10^5$  CD117+/CD34+ cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu per cell, ~~as for Fig-8.~~ Twenty-four hours post infection, the percentage of GFP positive was determined by flow cytometry. For mock infection, CD117+/CD34+ cells were incubated with virus dilution buffer only. The infections were done in triplicates, and the average percentage of GFP-expressing cells is indicated on the corresponding histogram. The SEM was less than 10% of the statistical average.

Please amend the paragraph at page 14, line 27 to read as follows:

-- Fig. 27 shows the substitution of the G-H loop with heterologous peptides (SEQ ID NOs.: 14-18). --

Please amend the paragraph at page 27, lines 13-30 continuing to page 28, lines 1-2, to read as follows:

-- To broaden the repertoire of cell types that Ad vectors can infect, a specific binding region, the G-H loop, within the knob domain has been newly identified herein to improve binding affinity and specificity. Alteration within this region will redirect the Ad vector to a desired cell type. For example, the invention describes the G-H loop sequence within the fiber protein knob domain, which can be replaced with heterologous peptide ligand sequences without affecting the functionally important tertiary structure of the Ad fiber knob domain, while changing the binding affinity and specificity ~~specifiety~~ of the

vector (Figure 27 Figs. 6,7). This G-H loop region is exposed on the central part of the knob surface and may be strategically a better site for incorporation of heterologous ligands than the peripheral H-I loop (Krasnykh, V. et al., 1998, J. Virol., 72:1844-52.) of the knob C-terminus (Michael, S. I., et al., 1995, Gene Ther., 2:660-8., Wickham, T. J. et al., 1996, Nat. Biotechnol., 14:1570-3.), which are the substitution sites used by others. Therefore, these G-H loop modifications within the fiber knob domain will allow the Ad vector to be redirected to infect a desired cell type, as long as the G-H loop ligand sequence binds to at least one surface protein on the desired cell type. Figure 27 Fig. 7 shows some possible substitutions. Example II J demonstrates that the virion tolerates the insertion of a cycling peptide (12 amino acids) with a constrained secondary structure that allows the exposure on the knob surface. A defined ligand (RGD) can be inserted into the G-H and the H-I loop of an Ad5 capsid that is ablated for CAR, and integrin tropism. Infectivity studies show the potential advantage of this new insertion site. --

Please amend the paragraph at page 33, lines 4-12, to read as follows:

-- Titers routinely obtained are in the range of  $3-8 \times 10^{12}$  genomes per ml. Assuming one genome is packaged per capsid, the genome titer equals the particle titer. The level of contaminating Ad.AAV1 is less than 0.1% as determined by Southern analysis, which is consistent with results obtained by plaque assay on 293 cells (fewer than 5 plaques per  $10^6$  total genomes). The primers used for sequencing the left and right ITR-vector-junction are

5'GGCGTTACTTAAGCTAGAGCTTATCTG (SEQ ID NO.:1), and  
5'CTCTCTAGTTCTAGCCTCGATCTCAC (SEQ ID NO.:2). --

Please amend the paragraph at page 73, lines 12-31, continuing to page 74, lines 1-23, to read as follows:

--  $1 \times 10^5$  CD34+ or K562 cells were infected in 100 $\mu$ l of growth media with different MOIs of Ad5, 9, or 35 which had been amplified in 293 cells, expressing the XhoI DNA methyltransferase isoshizomer PaeR7 (Nelson, J., Kay, M.A. 1997. *Journal of Virology*. 71:8902-8907). After 2 hours of incubation at 37°C, the cells were centrifuged at 1000 x g for 5 min, the virus-containing medium was removed, the cells were resuspended in 100 $\mu$ l of fresh media, and then they were incubated at 37°C until harvesting. At 16 hours post-infection for K562 cells, or 36 h post-infection for CD34+ cells, 5  $\mu$ g of pBS (Stratagene, La Jolla, CA) plasmid DNA was added as a carrier which could also be used as a loading control. Genomic DNA was extracted as described previously (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). One-fourth of purified cellular DNA (equivalent to  $2.5 \times 10^4$  cells) was digested with HindIII, XhoI, or with HindIII and XhoI together at 37°C overnight and subsequently separated in a 1% agarose gel followed by Southern blot with chimeric Ad5/9 or Ad5/35 DNA probes. The chimeric probes, containing sequences of Ad5 and Ad9 (Ad 5/9) or Ad5 and Ad35 (Ad 5/35), were generated by a two-step PCR amplification using Pfu-Turbo DNA polymerase (Stratagene, La Jolla, CA) and viral DNA from purified particles as a template. The following primers were used for PCR (Ad5 sequences and nucleotide numbers are underlined): Ad5F1—(nt: 32775-32805) 5' GCC CAA GAA TAA AGA ATC GTT TGT GTT ATG 3'; Ad5R1—(nt: 33651-33621) 5' AGC TGG TCT AGA ATG GTG GTG GAT GGC GCC A 3'; chimeric Ad5/9F—(nt: 31150-31177, nt: 181-208) 5' AAT GGG TTT CAA GAG AGT CCC CCT GGA GTC CTG TCA CTC AAA CTA GCT GAC CCA 3'; chimeric Ad5/9R—(nt: 32805-32775, nt: 1149-1113) 5' CAT AAC ACA AAC GAT TCT TTA TTC TTG GGC TTC ATT CTT GGG CGA TAT AGG AAA AGG 3'; chimeric Ad5/35F—(nt: 31150-31177, nt: 132-159) 5' AAT GGG TTT CAA GAG AGT CCC CCT GGA GTT CTT ACT TTA AAA TGT TTA ACC CCA 3'; chimeric Ad5/35R—(nt: 32805-32775, nt: 991-958) 5' CAT AAC ACA AAC GAT TCT

TTA TTC TTG GGC ATT TTA GTT GTC GTC TTC TGT AAT GTA AG-3' Ad5F1 –  
(nt: 32775-32805) 5'-GCC CAA GAA TAA AGA ATC GTT TGT GTT ATG-3' (SEQ  
ID NO.: 3); Ad5R1 – (nt: 33651-33621) 5'-AGC TGG TCT AGA ATG GTG GTG GAT  
GGC GCC A-3' (SEQ ID NO.:4); chimeric Ad5/9F – (nt: 31150-31177, nt: 181-208 )  
5'-AAT GGG TTT CAA GAG AGT CCC CCT GGA GTC CTG TCA CTC AAA CTA  
GCT GAC CCA -3' (SEQ ID NO.: 5); chimeric Ad5/9R – (nt: 32805-32775, nt:1149-  
1113) 5'- CAT AAC ACA AAC GAT TCT TTA TTC TTG GGC TTC ATT CTT GGG  
CGA TAT AGG AAA AGG-3' (SEQ ID NO.:6); chimeric Ad5/35F – (nt: 31150-31177,  
nt: 132-159) 5'-AAT GGG TTT CAA GAG AGT CCC CCT GGA GTT CTT ACT TTA  
AAA TGT TTA ACC CCA-3' (SEQ ID NO.:7), chimeric Ad5/35R (nt: 32805-32775,  
nt: 991-958) 5'-CAT AAC ACA AAC GAT TCT TTA TTC TTG GGC ATT TTA GTT  
GTC GTC TTC TGT AAT GTA AG-3' (SEQ ID NO.:8). Nucleotide numbers are given  
according to the sequences obtained from the NCBI GenBank (accession No. M73260 /  
M29978 for Ad5, X74659 for Ad9, and U10272 for Ad35). After the first amplification,  
the 968 bp-long Ad9, a 859 bp-long Ad35 DNA fragments corresponding to the fiber  
genes, and a 876 bp-long Ad5 fragment corresponding to the Ad5 E4 region (located  
immediately downstream of Ad5 fiber gene) were purified by agarose gel electrophoresis.  
To generate chimeric DNA probes, amplified Ad5 DNA was mixed with Ad9 or Ad35  
fragments obtained during the first step of PCR, and subjected to a second PCR  
amplification using Ad5/9F or Ad5/35F primers and the Ad5R1 primer. The resulting  
Ad5/9 or Ad5/35 chimeric DNA fragments (see Fig. 15) were purified and their  
concentrations were measured spectrophotometrically. Corresponding chimeric DNA  
fragments were loaded as concentration standards on agarose gels or labeled with [<sup>32</sup>P]-  
dCTP and used as probes for Southern analysis. The number of viral genomes per DNA  
sample was calculated after quantitative Phospho-imager analysis. In preliminary  
experiments, no preferential hybridization of chimeric DNA probes to DNA of any  
particular viral serotype was detected. --



Please amend the paragraph at page 83, lines 14-31 continuing to page 84, lines 1-19, to read as follows:

-- For transduction studies, two Ad vectors were constructed: Ad5GFP and Ad5GFP/F35, containing a chimeric Ad5/35 fiber gene. Both adenoviral vectors contained a 2.3kb, CMV promoter driven EGFP gene [derived from pEGFP-1, (Clontech, Palo Alto, CA)] inserted into the E3 region of Ad5. The EGFP expression cassette was cloned between Ad5 sequences 25,191-28,191 and 30,818-32,507 into a shuttle plasmid, which contained the E3 deletion described for pBHG10 (Microbix, Toronto, Canada). The resulting plasmid was named pAdGFP. For the chimeric vector, the Ad5 fiber gene in pAdGFP was substituted by an Ad5/35 chimeric fiber gene generated by the two-step PCR protocol outlined above. In the first PCR step, three DNA fragments corresponding to i) the Ad5 fiber 5'-nontranslated region and the first 132 bp of the fiber tail domain (nt 30,818-31,174), ii) the Ad35 shaft and knob domains (nt 132-991), and iii) the Ad5 E4 region including the Ad5 fiber polyadenylation signal (nt 32,775-33,651) were amplified by Pfu-Turbo DNA polymerase. The following primers were used: for the Ad5 tail, Ad5F-2 (nt 30,798-30,825) 5'-CGC GAT ATC GAT TGG ATC CAT TAA CTA-3' (SEQ ID NO.: 9) and Ad5R-2 (nt 31,174-31,153) 5'-CAG GGG GAC TCT CTT GAA ACC CAT T-3' (SEQ ID NO.: 10); for the Ad35 shaft and knob, primers Ad5/35F and Ad5/35R (see above); for the Ad5E4 and polyA, primers Ad5F-1 and Ad5R-1 (see above). After 10 PCR cycles, the products were purified by agarose gel electrophoresis, combined, and then subjected to a second PCR with primers Ad5F-2 and Ad5R-1. The resulting 2115 bp-long chimeric fiber gene contained the Ad5 tail and the Ad35 shaft and knob domains. This product was used as a substitute for the SalI/XbaI Ad5 fiber gene containing fragment in pAdGFP. The resulting plasmid was named pAdGFP/F35. To generate full-length E1/E3 vector genomes, pAdGFP and pAdGFP/F35 were inserted in pAdHM4 (Mizuguchi, H., Kay, M.A. 1998. *Human Gene Therapy*. 9:2577-2583) by recombination in *E.coli* (Chartier, C., E. et al. 1996. *Journal of Virology*. 70:4805-4810). To do this, the RecA+ *E.coli* strain BJ5183 was co-transformed with pAdHM4 linearized

by SrfI mixed with the XbaI fragments containing the GFP genes, the Ad5 or Ad5/35 fiber genes, and the Ad5 homology regions. The resulting recombinants were analyzed by restriction analysis. Correct recombinants were amplified in *E.coli* HB101 and purified by double CsCl gradient banding. The plasmids were named pAd5GFP and pAd5GFP/F35. The correct structure of the Ad5/35 chimeric fiber gene was confirmed by endonuclease digestion and sequencing part of pAd5GFP/F35. To produce the corresponding viruses, pAd5GFP and pAd5GFP/F35 were digested with PacI to release the viral genomes and transfected onto 293 cells as described (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). Plaques developed 7 to 10 days post-transfection in overlaid cultures. Recombinant viruses were propagated in 293 cells and purified by standard methods described elsewhere (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). --

Please amend the paragraph at page 95, lines 5-17 to read as follows:

-- Another alternative to make Ad5-capsid-based vectors suitable for HSC gene therapy is to incorporate the coding sequence for HSC specific peptides into the H1 loop region of the Ad5 fiber gene. The modification of the H1-loop was successfully exercised by Krasnykh et al. with a 7 amino-acid long FLAG peptide (DYDDDDK) (SEQ ID NO.: 11). Using phage display peptide libraries (Pascqualini, R. et al., 1996, *Nature*, 380:364-66), Renata Pasqualini (La Jolla Cancer Research Center) reported recently, at the First Meeting of the American Society for Gene Therapy, the identification of small peptide ligands specific for bone marrow cells. The corresponding sequences encoding these peptides can be added to modify the H1 loop sequence employing site-directed mutagenesis. Optimally, the ligands should allow for the efficient internalization of adenoviral particles based on a CAR- and integrin independent pathway. Modified adenoviral vectors containing the AAVBG cassette can be produced and tested for HSC tropism as described above. --

Please amend the paragraph at page 97, lines 2-27 to read as follows:

-- An example of a G-H loop substitution to target Ad5 to hepacytes was successful. Preliminary tests demonstrated that two evolutionarily conserved regions within the malaria circumsporozoite surface protein (CS) termed RI and RII+ mediate specific interaction with hepatocytes but not with other organs (including spleen, lung, heart and brain), nor with Kupffer cells, liver endothelial cells or with other regions of the hepatocyte membrane (Cerami, C. et al., 1992, *Cell*, 70:1021-33; Shakibaei, M. and U. Frevert, 1996, *J. Exp. Med.*, 184:1699-711). These regions are conserved among different species including *Plasmodium berghei*, *P. cynomogli*, and *P. falciparum* that infect mouse, monkey and human hepatocytes, respectively (Cerami, C. et al., 1992, *Cell*, 70:1021-33; Chatterjee, S. et al., 1995, *Infect Immun.*, 63:4375-81). Peptides derived from RI (KLKQPG) (SEQ ID NO.: 12) or RII (EWSPCSVTGNGIQVRIK) (SEQ ID NO.: 13) blocked CS binding to hepatocytes and infection by sporozoites in vivo ((Cerami, C. et al., 1992, *Cell*, 70:1021-33; Chatterjee, S. et al., 1995, *Infect Immun.*, 63:4375-81). RI and RII+ peptides were separately inserted into Ad5-fiber knob (H-I and G-H loop) containing mutation with abolished binding to CAR and alpha-v integrins (Kirby, L. et al., 2000, *J. Virol.*, 74:2804-13; Wickham, T. J. et al., 1995, *Gene Ther.*, 2:750-6). Based on preliminary data, a short-shafted fiber was used so that the virus entry strategy predominantly depends on the interaction with the primary (hepatocyte-specific receptor). The hepatocyte-specific ligands are flanked by short glycine stretches to provide flexibility and embedded into a loop formed by two cystines. This is one of the classical strategies to incorporate ligands into a protein scaffold (Doi, N. and H. Yanagawa, 1998, *Cell Mol. Life Sci.*, 54:394-404; Koivunen, E. et al., 1995, *Biotechnology (NY)*, 13:265-70) and to guarantee their presentation at the protein surface. The biodistribution of the best variants is tested in vivo in C57B1/6 mice based on Southern blots or PCR for vector DNA in different organs. This mouse strain is known to be susceptible to infection with *P. berghei* (Chatterjee, S. et al., 1995, *Infect Immun.*, 63:4375-81). --